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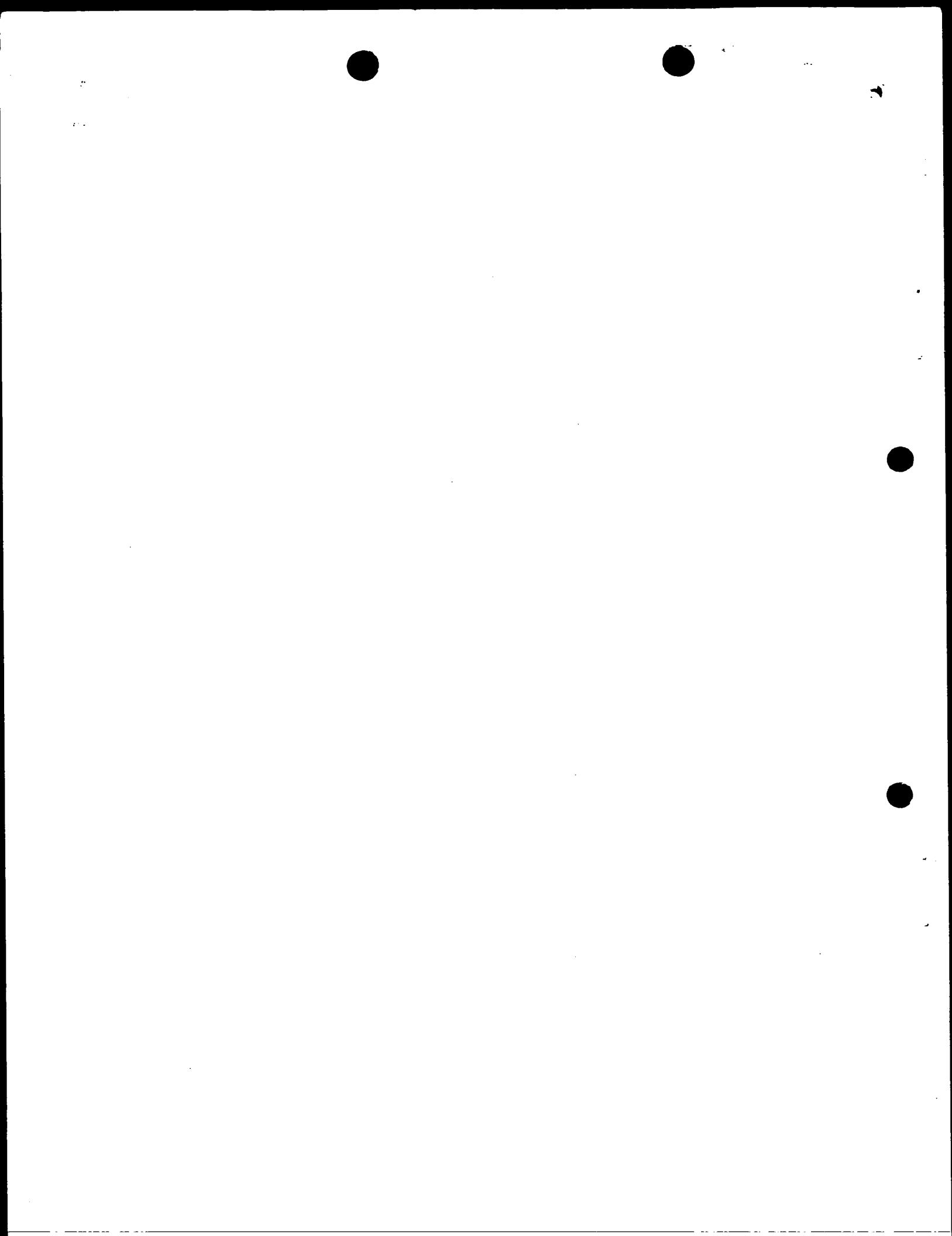
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## NEURITE REGENERATION

This invention relates to the regeneration of neurites and more particularly to the stimulation of neurite outgrowth in the peripheral and central nervous systems.

### INTRODUCTION

Retinoids are a family of molecules derived from vitamin A and include the biologically active metabolite, retinoic acid. The cellular effects of RA are mediated through the action of two classes of receptors, the retinoic acid receptors (RARs) which are activated both by *all-trans*-RA (*t*RA) and *9-cis*-RA (*9-cis*-RA), and the retinoid X receptors (RXRs), which are activated only by *9-cis*-RA (Kastner et al., 1994; Kleiwer et al., 1994). The receptors are of three major subtypes,  $\alpha$ ,  $\beta$  and  $\gamma$ , of which there are multiple isoforms due to alternative splicing and differential promoter usage (Leid et al.). The RARs mediate gene expression by forming heterodimers with the RXRs, whilst the RXRs can mediate gene expression as homodimers or by forming heterodimers with a variety of orphan receptors (Mangelsdorf & Evans, 1995). Many studies on a variety of embryonic neuronal types have shown that RA can stimulate both neurite number and length (review, Maden, 1998). as, indeed, can the neurotrophins (Campenot, 1977; Lindsay, 1988; Tuttle and Mathew, 1995). The neurotrophins are a family of growth factors that are required for the survival of a variety of neurons of primary sensory neurons in the developing peripheral nervous system (Snider, 1994). One of the earliest genes induced by NGF in PC12 cells is the orphan receptor NGFI-B

(NURR1) (Millbrandt, 1989). This suggests that the growth factor and retinoid mediated pathway in developing neurons can interact.

We have used embryonic mouse DRG to investigate further the nature of the interaction between the retinoid mediated pathway and the growth factor pathway to determine which of the RARs and RXRs are expressed in neurons that are dependent upon different neurotrophins for their survival. We have shown that RAR $\beta$ 2 is up-regulated in neurons that respond to RA by increasing neurite outgrowth and by the use of receptor selective agonists only the RAR $\beta$  agonist will substitute for RA in inducing neurite outgrowth. These results therefore indicate a role for RA acting via RAR $\beta$ 2 in the outgrowth of neurites from certain classes of neurons.

In the procedures described throughout this specification the materials used are either described in the literature, or available commercially, or available on request from the Developmental Biology Research Centre, The Randall Institute, Kings College, 26-29 Drury Lane, London WC2B 5RL, UK. (M.Maden or J.Corcoran). The primers used in pcr procedures are described in the Appendix given at the end of this specification.

#### MATERIALS & METHODS

DRG cultures : DRG were obtained from E13.5 mice, freed of non-ganglionic tissue and collected in ice-cold calcium magnesium free phosphate buffered saline. To prepare dissociated cell suspensions the ganglia were treated with 0.05 % trypsin for 15 minutes at 15 °C. The reaction was stopped by the addition of 1% serum and single cells obtained by trituration with a 23 G needle. The cells were then spun at 1000 g for ten minutes and resuspended in media. They were plated out at

a density of approximately of 25000 cells/cm<sup>2</sup> in wells that had been precoated first with 100 $\mu$ g/ml poly D lysine for 2 hrs and secondly with 100 $\mu$ g/ml laminin for 30 minutes. The cultures were fed every 2 days.

Culture media consisted of DMEM-F12 with glutamine (Gibco), 6 % glucose, ITS (Gibco). The growth factors used were either 50ng/ml NGF (7s, Promega) 50ng/ml NT3(promega) or 50ng/ml BDNF. Retinoids were used at a concentration of 1 x 10<sup>-7</sup> M. CD366 activates RAR $\alpha$ , CD2019 activates RAR $\beta$ , CD437 activates RAR $\gamma$  and CD2809 activates all of the RXRs.

RT-PCR analysis : RNA was extracted (trizol, Gibco) and cDNA prepared by the use of a pharmacia kit as described in the manufacturers instructions. The primers used were from mouse RARs RXRs and GAPDH (details upon request). PCR was carried out for 30 cycles. Amplification was carried out as follows, denaturation for 30 s at 95 °C, annealing for 30 s at 55°C and extension for 1 min at 72°C. One fifth of the resultant product was then run on a gel and blotted. This was then probed with the appropriate RAR and RXR and GAPDH for normalisation.

#### Human treatments

From the experiments we describe below we conclude that the upregulation and activation of RAR $\beta$ 2 is essential for neurite outgrowth.

## RESULTS

#### Response to RA

Neurons were grown in serum free medium for a period of two days before adding 1 x 10<sup>-7</sup>M RA to the cultures. The cultures were then fixed

three days later and analysed for neurite out growth with the monoclonal antibody NF200. In the absence of RA the BDNF neurons (Fig. 1 E) grew neurites whilst the NGF and NT-3 neurons (Fig. 1 A, C) showed little or no neurite outgrowth. In contrast when RA was added to the medium there was a increase in the length and number of neurites in the NGF and NT-3 neurons (Fig. 1 B, D) whilst RA did not effect the BDNF neurons (Fig. 1 F).

#### Expression of receptors and response to RA by RT-PCR

There was no difference in the expression of the RXRs in neurons cultured either in serum free medium or serum free plus RA (data not shown). In contrast there were variations in the RAR receptor profiles. Each of the three types of neurons expressed RAR $\alpha$  1 (Fig. 2 A, B, C, lane 1) and this receptor was up-regulated strongly in response to RA in the NGF and NT-3 neurons and slightly up-regulated in the BDNF neurons (Fig. 2 A, B, C, lane 8). Of the four possible RAR $\beta$  isoforms only the RAR $\beta$ 2 isoform was detected in each of the three types of neurons (Fig. 3 A, B, C, lane 2) and this was strongly upregulated by RA in the NGF and NT-3 neurons (Fig. 3 A, B, lane 6) but not in the BDNF neurons (Fig. 3 C, lane 6). The only RAR $\gamma$  isoform detected in the neuronal cultures was RAR $\gamma$ 1. This was strongly expressed in the NGF and NT-3 neurons (Fig. 4 A, B, lane 1) and was found to be down regulated in response to RA (Fig. 4 A, B, lane 8). No RAR $\gamma$  isoform was detected in the BDNF neurons either in the absence or presence of RA (data not shown).

#### Receptor selective analogues and neurite outgrowth

This data demonstrated that upon RA addition BDNF neurons were unaffected whereas NGF and NT-3 neurons up-regulated RAR $\beta$ 2 and

down-regulated RAR $\gamma$ 1. This suggested that the up-regulation and activation of RAR $\beta$ 2 is responsible for the increase of neurite outgrowth that we saw in the NGF and NT-3 neurons (Fig. 1 B, D). In order to test this hypothesis we used receptor selective syntheticretinoids which have been developed specifically to activate individual receptors. CD366 activates RAR $\alpha$ , CD2019 activates RAR $\beta$ , CD437 activates RAR $\gamma$  and CD2809 activates all of the RXRs.

The RAR $\beta$  agonist increased neurite outgrowth in the NGF and NT-3 neurons (Fig. 5 B, F), but did not effect neurite outgrowth in the BDNF neurons (Fig. 5 J). None of the other agonists increased neurite outgrowth in any of the neuronal populations (Fig. 5 A, C, D E, G, H, I, K, L).

## FIGURE LEGENDS

Figure 1. Effect of RA on neurite outgrowth from DRG neurons. DRG neurons were cultured either in the presence of NGF, NT-3 or BDNF for a period of two days at which point  $1 \times 10^{-7}$  M RA was added. They were then stained for neurite outgrowth after five days with NF200 antibody. A. NGF B NGF +  $1 \times 10^{-7}$  M RA. C. NT-3, D. NT-3 +  $1 \times 10^{-7}$  M RA. E. BDNF F. BDNF +  $1 \times 10^{-7}$  M RA

Figure 2. Expression of RAR $\alpha$  isoforms in DRG neurons cultured either in the absence or presence of RA. DRG neurons were cultured in the presence of either NGF, NT-3 or BDNF for a period of two days,  $1 \times 10^{-7}$  M RA was then added and the presence of the RAR $\alpha$  isoforms were then assayed by RT-PCR. A. NGF neurons B. NGF neurons +  $1 \times 10^{-7}$  M RA C. NT-3 neurons D. NT-3 neurons +  $1 \times 10^{-7}$  M RA E. BDNF neurons F. BDNF neurons +  $1 \times 10^{-7}$  M RA. Lanes: 1. RAR $\alpha$ 1 2. RAR $\alpha$ 2 3. RAR $\alpha$ 3 4. RAR $\alpha$ 4 5. RAR $\alpha$ 5 6. RAR $\alpha$ 6 7. RAR $\alpha$ 7

Figure 3. Expression of RAR $\beta$  isoforms in DRG neurons cultured either in the absence or presence of RA. DRG neurons were cultured in the presence of either NGF, NT-3 or BDNF for a period of two days,  $1 \times 10^{-7}$  M RA was then added and the presence of the RAR $\beta$  isoforms were then assayed by RT-PCR. A. NGF neurons B. NGF neurons +  $1 \times 10^{-7}$  M RA C. NT-3 neurons D. NT-3 neurons +  $1 \times 10^{-7}$  M RA E. BDNF neurons F. BDNF neurons +  $1 \times 10^{-7}$  M RA. Lanes: 1. RAR $\beta$ 1 2. RAR $\beta$ 2 3. RAR $\beta$ 3 4. RAR $\beta$ 4

Figure 4. Expression of RAR $\gamma$  isoforms in DRG neurons cultured either in the absence or presence of RA. DRG neurons were cultured in the presence of either NGF, NT-3 or BDNF for a period of two days,  $1 \times 10^{-7}$  M RA was then added and the presence of the RAR $\gamma$  isoforms were then assayed by RT-PCR. A. NGF neurons B. NGF neurons +  $1 \times 10^{-7}$  M RA C. NT-3 neurons D. NT-3 neurons +  $1 \times 10^{-7}$  M RA E. BDNF neurons F. BDNF neurons +  $1 \times 10^{-7}$  M RA. Lanes: RAR $\gamma$ 1 2 RAR $\gamma$ 2 3 RAR $\gamma$ 3 4. RAR $\gamma$ 4 5. RAR $\gamma$ 5 6. RAR $\gamma$ 6 7. RAR $\gamma$ 7

Figure 5. Effect of RAR and RXR agonists on neurite outgrowth from DRG neurons. DRG neurons were cultured either in the presence of NGF, NT-3 or BDNF for a period of two days at which point either  $1 \times 10^{-7}$  M RA or a RAR $\alpha$ , RAR $\beta$ , RAR $\gamma$  or RXR agonist were added to the cultures. Cultures were then stained for neurite outgrowth at five days with the NF200 antibody. A- D NGF type neurons E-H NT-3 type neurons I-J BDNF type neurons, agonists RAR $\alpha$  A, E I, RAR $\beta$ , B, F, J., RAR $\gamma$  C, G, K, RXR D, H, L.

Nerve growth factor (NGF) stimulates neurite outgrowth from cultured adult dorsal root ganglia (DRG). The vitamin A derivative all-*trans*-retinoic acid (tRA) induces neurite outgrowth from various embryonic sources, including DRG. Here we show that NGF is upstream of tRA. When adult mouse DRG are cultured in the presence of NGF and an inhibitor of tRA synthesis, neurite outgrowth does not occur. Conversely, when tRA is added along with a blocking antibody to NGF, neurite outgrowth occurs as normal. We further show that NGF induces transcription of both the tRA-synthesizing enzyme RALDH-2 and the retinoic acid receptor- $\beta$ 2 as well as detectable release of synthesized tRA. We deduce that tRA is required for adult DRG neurite regeneration and that NGF acts upstream of RA to induce its synthesis.

## METHODS

DRG cultures: whole DRG were obtained from adult mice of approximately six months old. These were cultured in cellogen matrix (ICN flow), prepared by mixing 1 volume of 7.5 % sodium bicarbonate, 1 volume of 10x MEM (Gibco) and 8 parts cellogen (ICN flow). The pH was adjusted to 7.5 by dropwise addition of 5M NaOH. The cultures were fed every 2 days. Culture media consisted of DMEM-F12 with glutamine (Gibco), 6 % glucose, GMS-A (Gibco) and 10% delipidated serum. The supplements used were 100ng/ml NGF (7s, Promega),  $1 \times 10^{-7}$  M RA (sigma),  $1 \times 10^{-5}$  M disulphiram, 1/500 dilution of blocking antibody against NGF (chemicon); and dimethyl sulphoxide. Cultures were fed every two days, on the fifth day they were fixed in 4 % paraformaldehyde and stained with the neurofilament antibody, NF200 (sigma). In parallel

experiments the DRG were either collected for PCR analysis or they were sonicated before been placed on the F9 reporter cells.

RT-PCR analysis: RNA was extracted (trizol, Gibco) and cDNA prepared by the use of a pharmacia kit as described in the manufactures instructions. The primers used were from mouse RALDH-2, mouse GAPDH and mouse RARB (details upon request). PCR was carried out for 30 cycles for RALDH-2 and 25 cycles for RAR $\beta$ 2. Amplification was carried out as follows, denaturation for 30 s at 95°C, annealing for 30 s at 55°C and extension for 1 min at 72°C. One fifth of the resultant product was then run on a gel and blotted. This was then probed with cDNAs coding either for RALDH-2 or RAR $\beta$ 2 and GAPDH for normalisation.

Reporter cell assay: the generation and characterisation of the F9 reporter cells has been described<sup>21</sup> For the assay the cells were grown on gelatin coated wells of a 24-well culture dish (Nuclon) to confluence. Just prior to introducing the sonicated DRG to the wells the growth medium was aspirated from the F9 cells and replaced with 0.5ml serum-free medium. After overnight incubation the cultures were fixed and the  $\beta$ -galactosidase expression was visualised. They were gently washed twice with room temperature PBS, then fixed for 5 minutes at 4°C with 2% paraformaldehyde, 0.2% glutaraldehyde in PBS. They were rinsed gently twice more then covered with 1ml of X-gal stain per dish and incubated at 37°C overnight. The X-gal stain was replaced with PBS and the blue stained cells counted on an Olympus inverted microscope fitted with an eyepiece graticule. The cells were counted in three random fields for each of the three experiments.

### Human treatment

Retinoids that are RAR $\beta$ 2 agonists can be applied topically over the damaged area or given orally. A suitable dose is in the range 0.025 %-0.1% in creams or given orally at 1-2 mg/kg body weight.

### RESULTS

Adult mouse DRG were cultured in the presence of NGF (100ng per ml), or tRA (100 nM) for five days. In both cases neurite outgrowth occurred (Fig. 6b and data not shown). Little or no neurite outgrowth occurred in adult DRG cultured in only delipidated serum (Fig. 6a). Differences in number of neurites were significant (Fig. 7a; 1, 2 and 3). When tRA was added together with NGF, there was no additive effect of the two treatments (Fig. 6c), and no significant difference was seen between tRA, NGF or tRA plus NGF groups (Fig. 7a; 2, 3 and 4).

To show that NGF induces RA we cultured adult DRG in the presence of NGF and 10  $\mu$ M disulphiram, a compound which blocks the conversion of retinaldehyde to tRA by inhibiting the enzyme retinaldehyde dehydrogenase. As shown in Fig. 6d, addition of disulphiram completely abolished NGF-induced neurite outgrowth compared to NGF alone (Fig. 7a, 2 and 5) or NGF plus DMSO (vehicle for disulphiram) (Fig. 7a, 2 and 6). To confirm that disulphiram did not affect cell survival within the explants, we performed two types of rescue. Explants were cultured for eight days in medium supplemented with disulphiram. In the first rescue, tRA was added to the explants from the beginning of the experiment; in the second, tRA was added on day 4. Neurite outgrowth occurred in both of the rescued cultures and not in the disulphiram alone cultures (Figs. 6e, f and 7b).

Inhibition of the inductive effect of NGF but not of tRA by disulphiram suggests that NGF may precede tRA in the cascade leading to neurite outgrowth. To test this, we used a blocking antibody against NGF. In the presence of NGF and the blocking antibody, virtually no neurite outgrowth occurred (Fig. 6g; compare to DRG cultured in the presence of NGF alone, Fig. 6b). On the other hand, DRG cultured in the presence of the NGF-blocking antibody and tRA (Fig. 6h) showed neurite outgrowth equivalent to that obtained with NGF alone (Figs. 6b and 7c).

If NGF is upstream of RA, it should induce synthesis of tRA after addition to DRG cultures. To test this we used an F9 reporter cell line that responds to the presence of tRA due to transfection with 1.8 kb of the mouse RAR $\beta$ 2 gene promoter containing a retinoic acid response element linked to the *lacZ* gene (Maden, M., Sonneveld, E., van der Saag, P. T. & Gale, E. *Development* 125, 4133–4144 (1998)). In the presence of tRA, activated cells can be detected after  $\beta$ -galactosidase histochemical staining. NGF itself does not activate these cells because in the presence of NGF there was no labeling of the F9 cells above background. We then cultured adult DRG in delipidated serum for five days under three different conditions: no NGF, with NGF or NGF plus the NGF-blocking antibody. The DRG were then sonicated and placed on the F9 reporter cells. NGF-treated DRG homogenates produced a clear RA signal relative to untreated DRG (Fig. 7d). This activation was prevented when the DRG were cultured with blocking antibody in addition to NGF (Fig. 7d).

We next showed which tRA synthesizing enzyme might be induced by NGF. Retinol is converted by a two-step oxidative process firstly to retinaldehyde and then to retinoic acid. It has been shown that retinaldehyde dehydrogenase type 2 (RALDH-2) is expressed in the developing nervous system. Using RT-PCR, we found strong induction of

RALDH-2 by NGF in cultured adult DRG (Fig. 7e). Lastly, we also found up-regulation of the RAR $\beta$ 2 receptor in NGF-stimulated cultures (Fig. 7e) which we have shown to be required for neurite outgrowth.

Our results show that tRA can stimulate neurite outgrowth from an adult neural tissue, the DRG. NGF similarly stimulates neurite outgrowth from this tissue, and we have demonstrated that it does so by inducing tRA synthesis via an enzyme, RALDH-2. The sequence of events in the induction of neurite outgrowth by NGF is: NGF ----> RALDH-2 ----> tRA ----> RAR $\beta$ 2 ----> neurite outgrowth.

Neurotrophins have been considered as potential agents for induction of nerve regeneration and treatment of neurodegenerative diseases, but a major problem for their use is lack of effective modes of delivery to the site of the injury. Because tRA is required for the regenerative response and it is downstream of NGF, then the problem of delivery could be overcome as tRA is a low-molecular-weight lipophilic compound that can be administered orally. Thus, tRA is proposed for clinical use in neurology.

## FIGURE LEGENDS

Figure 6. Neurite outgrowth in adult mouse DRG cultured for five (a-d, g, h) or eight days (e, f) in the presence of delipidated serum plus: (a) no addition; (b) NGF, 100 ng per ml; (c) NGF and 100 nM tRA; (d) NGF and 10 mM disulphiram; (e) disulphiram and tRA added on day 0; (f) disulphiram; (g) NGF and blocking antibody (h) NGF-blocking antibody and tRA.

Figure 7. Neurite numbers (a-c), tRA synthesis (d) and gene induction (e) in adult mouse DRG after various treatments. (a) Effects of NGF, tRA and disulphiram at five days (1, no additive; 2, NGF, 100 ng per ml; 3, tRA, 100 nM; 4, NGF, 100 ng per ml and tRA, 100 nM; 5, 100 ng/ml NGF and 10 mM disulphiram; 6, NGF, 100 ng per ml and DMSO). Error bars = s.e.;  $n = 6$ . \* $p < 0.01$ ; \*\* $p < 0.0001$ ; Student's *t*-test. (b) tRA rescue of DRG treated with 10 M disulphiram (1, no tRA; 2, 100 nM tRA on day 0; 3, 100 nM tRA on day 4).  $n = 6$ . (c) NGF-blocking antibody on 5-day DRG cultures. 1, NGF, 100 ng per ml; 2, NGF plus blocking antibody; 3, blocking antibody plus 100 nM tRA.  $n = 4$ . (d) Increase in percentage  $\beta$ -gal-positive F9 cells in response to cultured DRG. 1, no additive; 2, NGF, 100 ng per ml; 3, NGF plus blocking antibody.  $n = 9$ . (e) RT-PCR analysis of RALDH-2 enzyme and RAR $\beta$ 2 expression in adult DRG cultured with (lane 2) or without (lane 1) NGF (100ng per ml) for five days. GAPDH was used to indicate presence of cDNA in both samples.

We have demonstrated above that the retinoic acid receptor  $\beta 2$  is a crucial transducer of the retinoic acid signal to stimulate the outgrowth of neurites in embryonic and adult dorsal root ganglia. We have also discovered that in embryonic mouse spinal cord the same situation exists, RAR $\beta 2$  is up-regulated at concentrations which maximally stimulate neurite outgrowth. In the adult mouse spinal cord, however, no such up-regulation is observed and no neurites are extended *in vitro*. But when the adult cord is transfected with RAR $\beta 2$  then neurite outgrowth occurs. It does not occur when the cord is transfected with another isoform of RAR $\beta$ , RAR $\beta 4$  thus showing a high level of specificity of receptor function.

The failure of CNS axons to regenerate under normal circumstances has been attributed to one or a combination of causes: the low abundance of neurotrophic factors; the absence of growth-promoting molecules; the presence of growth-inhibiting molecules. However, in the following description we show that by manipulating the genome of the neurons themselves, a stimulation of neurite outgrowth can be obtained from adult mouse spinal cord *in vitro*. We have transfected the adult spinal cord with a nuclear transcription factor, the retinoic acid receptor- $\beta 2$  (GenBank ref. nos. x56573, MMRARB2), and as a result transformed the normally inert spinal cord into one which can extend neurites. Therefore, the manipulation of both the neuronal genome and the environment may lead to more complete spinal cord regeneration than either manipulation alone.

## METHODS

Cultures. Spinal cord was dissected from either E13.5 or 10 month old mice and cut into transverse pieces of about 5 mm. These were cultured in cellogen matrix (ICN flow), prepared by mixing 1 volume of 7.5 % sodium bicarbonate, 1 volume of 10x MEM (Gibco) and 8 parts cellogen (ICN flow). The pH was adjusted to 7.5 by dropwise addition of 5M NaOH. Explants were fed every two days. The media consisted of DMEM-F12 with glutamine (Gibco), 6 % glucose, GMS-A (Gibco) 10% delipidated serum and all-*trans*-RA (stock solution,  $1 \times 10^{-5}$  M, Sigma). On the fifth day they were fixed in 4 % paraformaldehyde and stained with the neurofilament antibody, NF200 (Sigma).

RT-PCR analysis. RNA was extracted (trizol, Gibco) and cDNA prepared by the use of a Pharmacia kit as described in the manufacturers instructions. The primers used were from GAPDH, RAR $\beta$ 2 and RAR $\beta$ 4. PCR was carried out for 30 cycles for embryonic spinal cord and 40 cycles for adult spinal cord. Amplification was carried out as follows, denaturation for 30 s at 95°C, annealing for 30 s at 55°C and extension for 1 min at 72°C. One fifth of the resultant product was then run on a gel.

Transfections. Virus stocks were prepared and *B* galactosidase staining carried out as described by Lim, F., Hartley, D., Starr, P., Song, S., Lang, P., Yu, L., Wang, Y.M. & Geller, A.I. Use of defective herpes-derived plasmid vectors. *Meth. Mol. Biol.* 62, 223-232 (1997).

The titres used were: pHSV RAR $\beta$ 2,  $5 \times 10^{-4}$  ip/ul, pHSVRAR $\beta$ 4,  $4 \times 10^{-4}$  ip/ul, pHSVlacZ  $5 \times 10^{-4}$  ip/ul.

### Human Treatment

The method of treatment involves insertion of the human RAR $\beta$ 2 cDNA into the defective herpes simplex viral vector. This is then placed at the site of injury by injecting the construct into both ends of the injured cord. The dose used is in the range of  $4 \times 10^{-4}$  -  $5 \times 10^{-4}$  ip/ $\mu$ l. The duration of the treatment will depend upon the severity of the injury.

### RESULTS

#### Effect of RA on embryonic mouse spinal cord in vitro.

E13.5 spinal cord was dissected from mouse embryos placed in acellogen matrix and cultured in 10 % delipidated serum. All-*trans*-RA was added at 3 different concentrations ( $10^{-8}$ M,  $10^{-7}$ M,  $10^{-6}$ M) and after 5 days the explants were stained with a neurofilament antibody and examined for the presence of neurites. There was an increasing number of neurites emerging from the cultured cord with increasing concentrations of RA with the maximal effect at  $10^{-6}$ M (Fig. 8C, E, G). To demonstrate that the induction of neurites involved the up-regulation of RAR $\beta$ 2, RT-PCR was performed on cultures after 5 days in the same range of RA treatments. This revealed that RAR $\beta$ 2 is normally expressed in embryonic spinal cord at all concentrations of RA used (Fig. 9A, lanes 1-5) and that it is strongly up-regulated after  $1 \times 10^{-6}$  M RA treatment (Fig. 9A, lane 5), the same concentration which gives maximal neurite outgrowth.

#### Lack of effect of RA on adult mouse spinal cord in vitro.

An identical series of experiments was performed using 10 month old adult spinal cord rather than the embryonic cord. In contrast to the embryonic cord, RA had no effect on neurite outgrowth at any concentration tested and like the untreated controls, these RA treated

adult cords failed to extend any neurites at all (Fig. 8B, D, F, H). Examining the involvement of RAR $\beta$ 2 by RT-PCR revealed that control adult spinal cord had little or no detectable endogenous levels of this receptor (Fig. 9B, lane 1) and that there was no change in its level in response to RA treatment at any concentration (Fig. 9, lanes 2-5), unlike the embryonic cord.

#### Induction of neurites in adult spinal cord

We have therefore concluded that it was the lack of RAR $\beta$ 2 expression which may be responsible for the completely inert behaviour of the adult spinal cord. To test this further we used a defective herpes simplex virus type 1 (HSV-1) vector to transfect pieces of adult (10 months) mouse spinal cord. Three different transfections were performed, two of which served as controls. Firstly, just the vector containing lacZ (pHSVlacZ); secondly the vector containing RAR $\beta$ 2 (pHSVRAR $\beta$ 2) ; thirdly the vector containing another isoform of the RAR $\beta$  gene, RAR $\beta$ 4 (pHSVRAR $\beta$ 4). The latter served as a very precise control for transfection since we have not detected the RAR $\beta$ 4 isoform after RA treatment of neurons in our previous experiments hence it is not involved in neurite outgrowth. We first ensured that the transfections were successful and that the relevant receptor isoform was expressed in the cultured cord. Pieces of spinal cord were transfected overnight with the appropriate construct and analysed either three or four days later. The pHSVlacZ treated cords showed a significant amount of transfection had taken place as judged by b-galactosidase staining of the adult cord (Fig. 10B). RT-PCR demonstrated that transfection with the RAR $\beta$ 2 vector resulted in the expression of RAR $\beta$ 2 (Fig. 11, lane 3) but not RAR $\beta$ 4 (Fig. 11, lane 4) and transfection with the RAR $\beta$ 4 vector resulted in the expression of RAR $\beta$ 4 (Fig. 11, lane 8) but not RAR $\beta$ 2 (Fig. 11, lane 7). In the non

transfected cord neither RAR $\beta$ 2 or RAR $\beta$ 4 were detected (Fig. 11, lanes 2 and 6).

The effects of these transfections on neurite outgrowth were clear-cut. Transfection with the pHHSVlacZ failed to change the behaviour of the cultured adult cord which remained completely un-responsive in terms of neurite outgrowth (Fig. 12A, 12/12 transfections). Similarly, the transfections with pHSVRAR $\beta$ 4 produced no response in the cultured cord which remained inert (Fig. 12C, 12/12 transfections). However, transfections with the pHSVRAR $\beta$ 2 isoform clearly produced a different behaviour and many neurites appeared in the cultures (Fig. 12B, 8/12 transfections). The number of neurites produced varied between 3 and 23. In the pHHSVlacZ transfections there was considerable variability in the number of lacZ-positive cells per explant. This suggests that the variability in neurite number may be due to variability in number of cells transfected.

## FIGURE LEGENDS

Figure 8. Comparison of the effect of retinoic acid on neurite outgrowth on cultured E13.5 (A, C, E, G) and 10 month old adult spinal cord (B, D, F, H). Pieces of spinal cord were cultured in cellogen in the presence of 10 % delipidated serum and RA for a period of five days. The medium was changed every two days. A, B, no RA; C, D,  $1 \times 10^{-8}$  M RA; E, F,  $1 \times 10^{-7}$  M RA; G, H,  $1 \times 10^{-6}$  M RA.

Figure 9. Expression of RAR $\beta$ 2 in E13.5 and 10 month old adult spinal cord. Pieces of spinal cord were cultured in the presence of various concentrations of RA for a period of five days after which time RT-PCR analysis of RAR $\beta$ 2 was performed. A. E13.5 (lanes 2-5) and B. 10 month old adult spinal cord (lanes 2-5). Lanes: 1. bluescript/HPA II size markers, 2. no RA, 3.  $1 \times 10^{-8}$  M RA, 4.  $1 \times 10^{-7}$  M RA, 5.  $1 \times 10^{-6}$  M RA. The presence of GAPDH was used to indicate equal amounts of cDNA in the samples.

Figure 10. Transfection of adult spinal cord with pHSVlacZ. Cultured 10 month old adult spinal cord was transfected with  $5 \times 10^{-4}$  ipu/ul pHSVlacZ overnight and analysed for *B* galactosidase staining 3 days later. A. non-transfected adult spinal cord. B. adult spinal transfected with pHSVlacZ.

Figure 11. Transfection of adult spinal cord with either pHHSVRAR $\beta$ 2 or pHSVRAR $\beta$ 4. Adult spinal cord was cultured in cellogen and transfected either with  $5 \times 10^{-4}$  ipu/ul of pHHSVRAR $\beta$ 2 or  $4 \times 10^{-4}$  ipu/ul pHSVRAR $\beta$ 4 overnight. RT-PCR analysis four days after transfection, of RAR $\beta$ 2 (lanes 2-4) and RAR $\beta$ 4 (lanes 6-8) expression in adult spinal cord

transfected with Lanes 2, 6 no virus , 3, 7 pHSVRAR $\beta$ 2, 4, 8, pHSVRAR $\beta$ 4. The presence of GAPDH was used to indicate equal amounts of cDNA in the samples. Lanes 1,2 bluescript/HPA II size markers.

Figure 12. Effect of either pHSVlacZ, pHSVRAR $\beta$ 2 or pHSVRAR $\beta$ 4 transfection in cultured adult spinal cord on neurite outgrowth. Ten month old spinal cord was cultured in cellogen and transfected with either  $5 \times 10^{-4}$  ipu/ $\mu$ l, pHSVlacZ,  $5 \times 10^{-4}$  ipu/ $\mu$ l, pHSVRAR $\beta$ 2 or  $4 \times 10^{-4}$  ipu/ $\mu$ l pHSVRAR $\beta$ 4 overnight, and analysed for neurite staining with NF200 4 days after transfection. Cultured spinal cord transfected with A. pHSVlacZ, B. pHSVRAR $\beta$ 2, C. pHSVRAR $\beta$ 4.

## CONCLUSION

These results strongly indicate that the RAR $\beta$ 2 isoform plays a key role in the induction of neurite outgrowth in response to RA and that this may be a crucial component which fails to be up-regulated in the injured adult CNS. Our conclusion is based upon several experiments involving either regenerating or non-regenerating neuronal tissues and their response to RA. Thus the embryonic mouse spinal cord, the embryonic mouse DRG and the adult mouse DRG all respond to RA by up-regulating RAR $\beta$ 2 and extending neurites. In contrast, the adult mouse spinal cord fails to up-regulate RAR $\beta$ 2 and fails to extend neurites. Furthermore, NGF stimulates neurite outgrowth and acts by up-regulating RAR $\beta$ 2 and neurite outgrowth from embryonic mouse DRG can be stimulated by a RAR $\beta$  agonist.

These results reveal that when the genome of the neuron itself is manipulated then regeneration can be reawakened. These data support a role of RAR $\beta$ 2 in the regeneration of neurites in the adult CNS and feasibility of gene therapy with this transcript in combination with other treatments to achieve functional recovery of the injured spinal cord.

The invention therefore comprises a method of treatment of neurodegenerative disease in which expression of the retinoic acid receptor RAR $\beta$ 2 is ensured in affected cells or tissues. This may be achieved by treatment with an agonist of the RAR $\beta$ 2 receptor or by gene therapy i.e. insertion of the nucleic acid coding for this receptor into the required site *in vivo*. The latter may be achieved by incorporation of the receptor cDNA in a suitable vector as in, for example, pHSV-RAR $\beta$ 2. The invention may also be seen as the use of these agents in medication for the treatment of peripheral nervous injuries and spinal cord regeneration e.g. in cases of paraplegia.

## APPENDIX

RARalpha reverse primer: 535 tggtagctcttgagcactc 517

RARalpha1 forward strand primer 648 tacgccttctttcccc 666

RARalpha2 forward strand primer 376 cttttataaccagaaccgggc 396

RARalpha3 forward strand primer 111 caagtagaaggccaggaaagtc 131

RARalpha4 forward strand primer 3 ctaagaagacccacactctg 23

RARalpha5 forward strand primer 30 aagtggatgtgaaaactggg 48

RARalpha6 forward strand primer 42 ttcacagcctggcataac 59

RARalpha6 forward strand primer 24 gagaaggaagtgagccatc 42

RARbeta reverse strand primer 508 tctctgtgcattcctgcttg 488

RARbeta 1 forward strand primer 180 tggacacatgactcactacc 199

RARbeta 2 forward strand primer 598 atgttctgtcagtggatccc 617

RARbeta 3 forward strand primer 457 gcatgtcagaggacaactg 475

RARbeta 4 forward strand primer 21 agcctggaaaatgccatc 38

RARGamma reverse strand primer 481 ttacagcttccttggacatgcc 460

RARGamma1 forward strand primer 119 agatgctgagccctagctc 138

RARGamma2 forward strand primer 73 ttactacgcagagccactgg 92

RARGamma3 forward strand primer 169 ggaagatggaagagggaaac 187

RARGamma4 forward strand primer 230 caaatttactgggggttgg 248

RARGamma5 forward strand primer 18 ggctggattttggattgaag 37

RARGamma6 forward strand primer 329 ttctgtcctctcactaccttgg 350

RARGamma7 forward strand primer 85 cattaccgcgagtcactaac 104

GAPDH

forward primer 37 cgtagacaaaatggtaagg 56

reverse primer 333 gactccacgacatactcagc 314

RALDHII

forward primer 1190 gcttcattcattgacccac 1208

reverse primer 1539 cttcaccgtcaggctttac 1519

RXRalpha

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reverse primer 994 tctagggcagctcagaaaag 974

RXRbeta

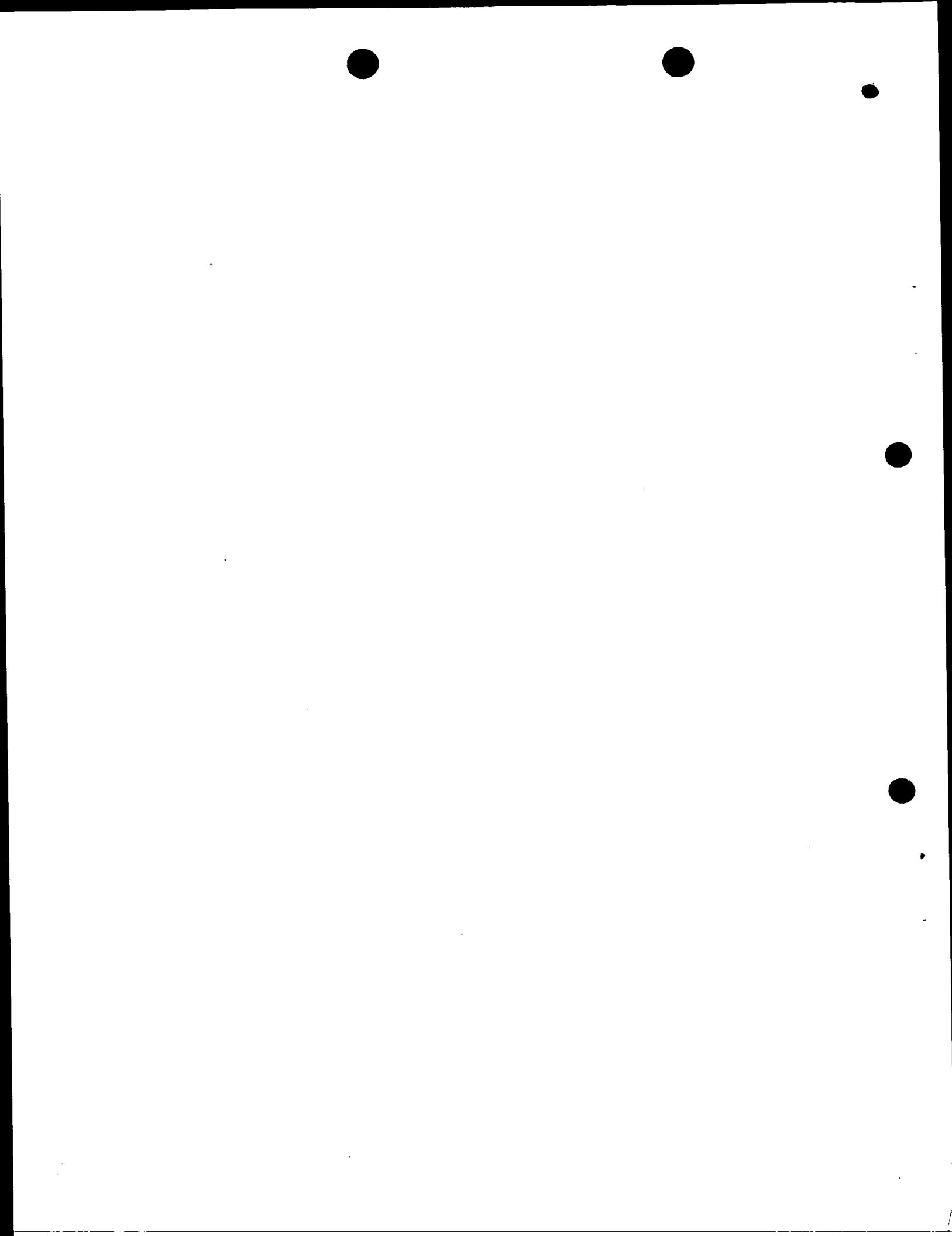
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reverse primer 2176 catcaatgtccccacttg 2159

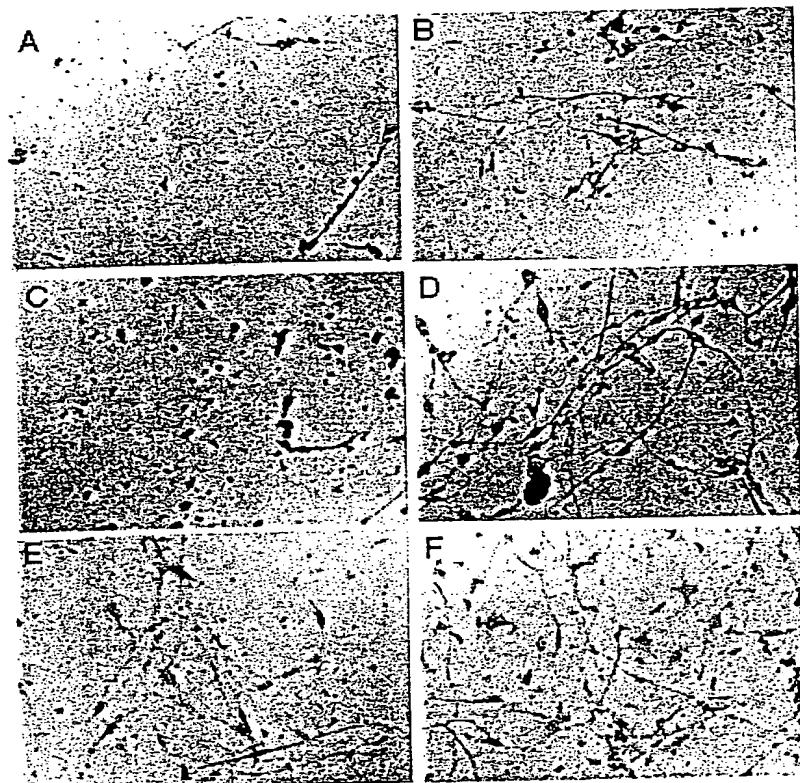
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○ Figure 1.



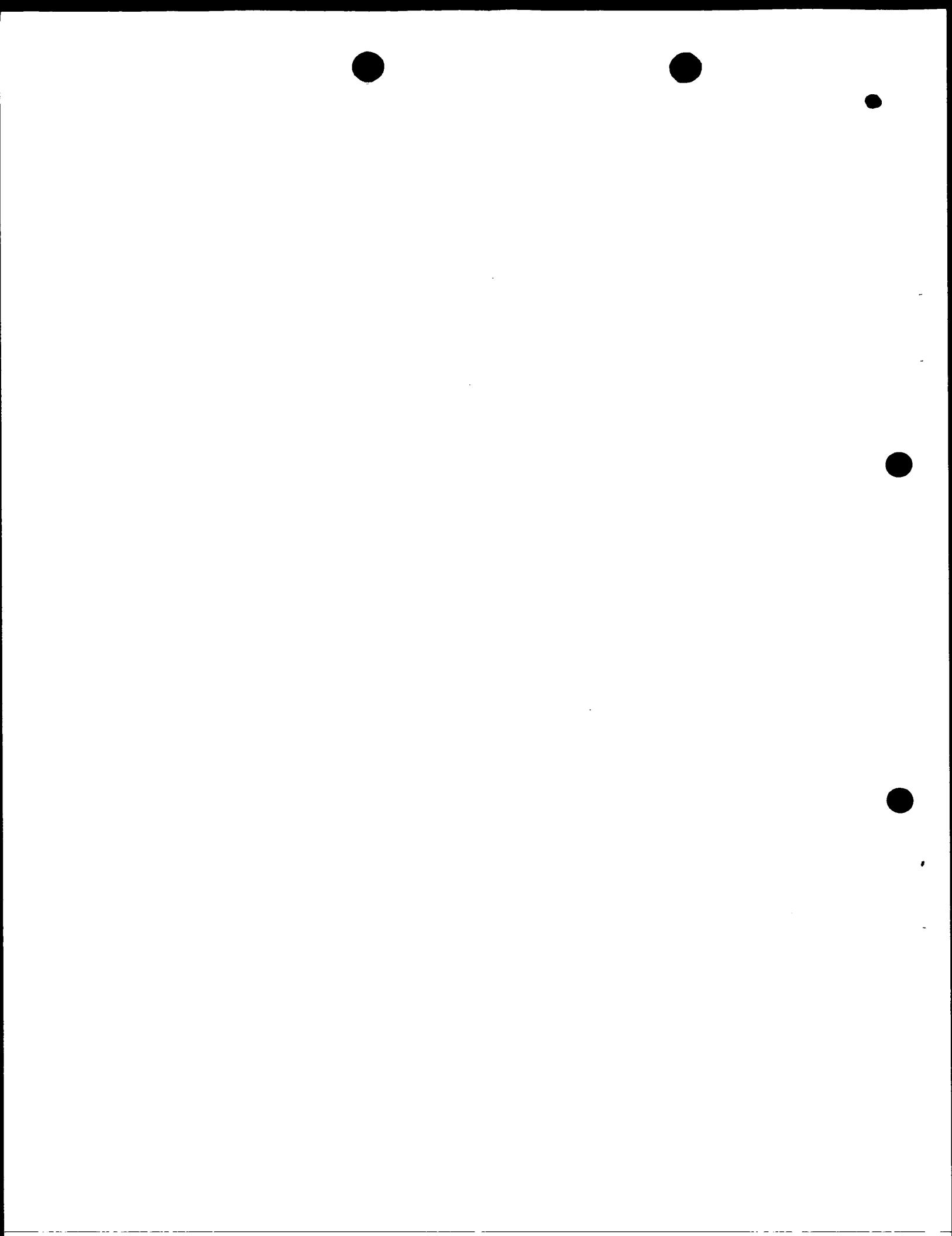


Figure 2.

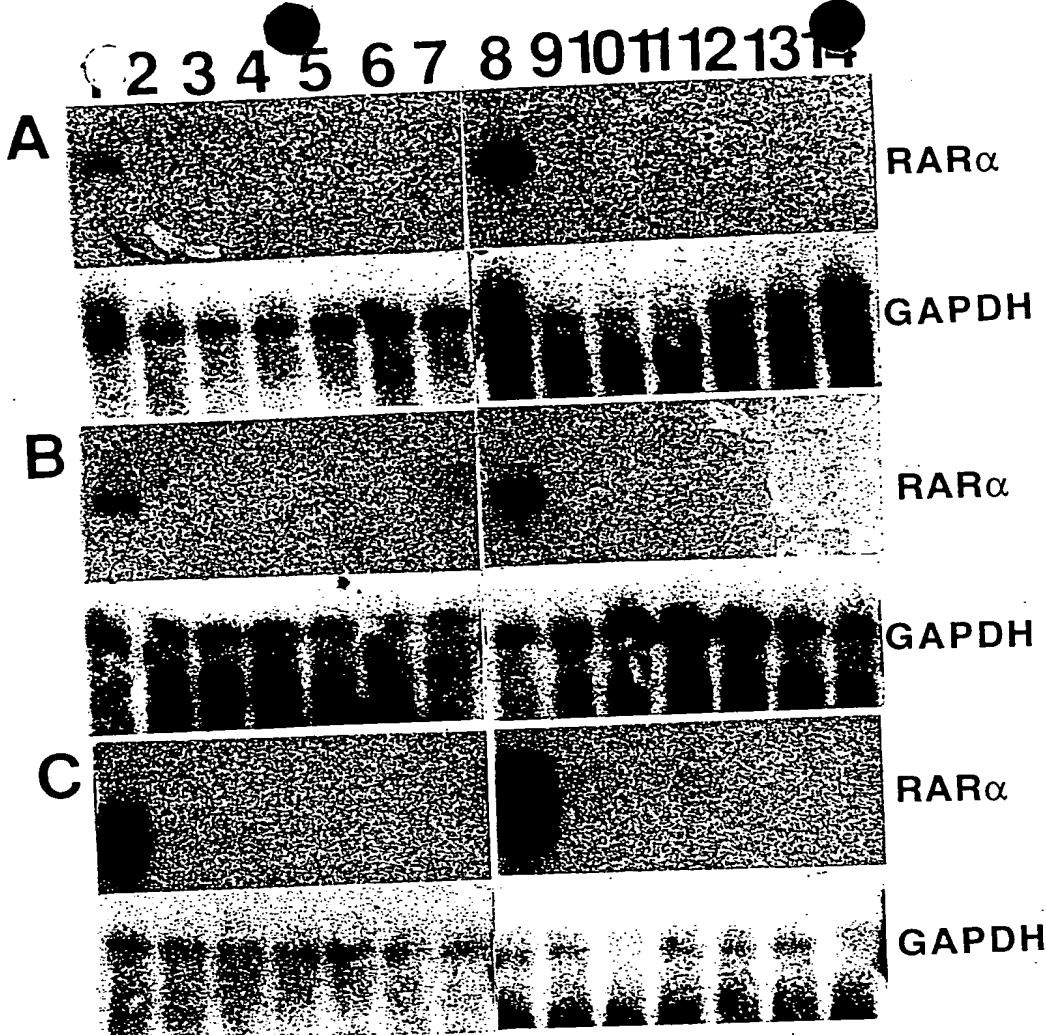
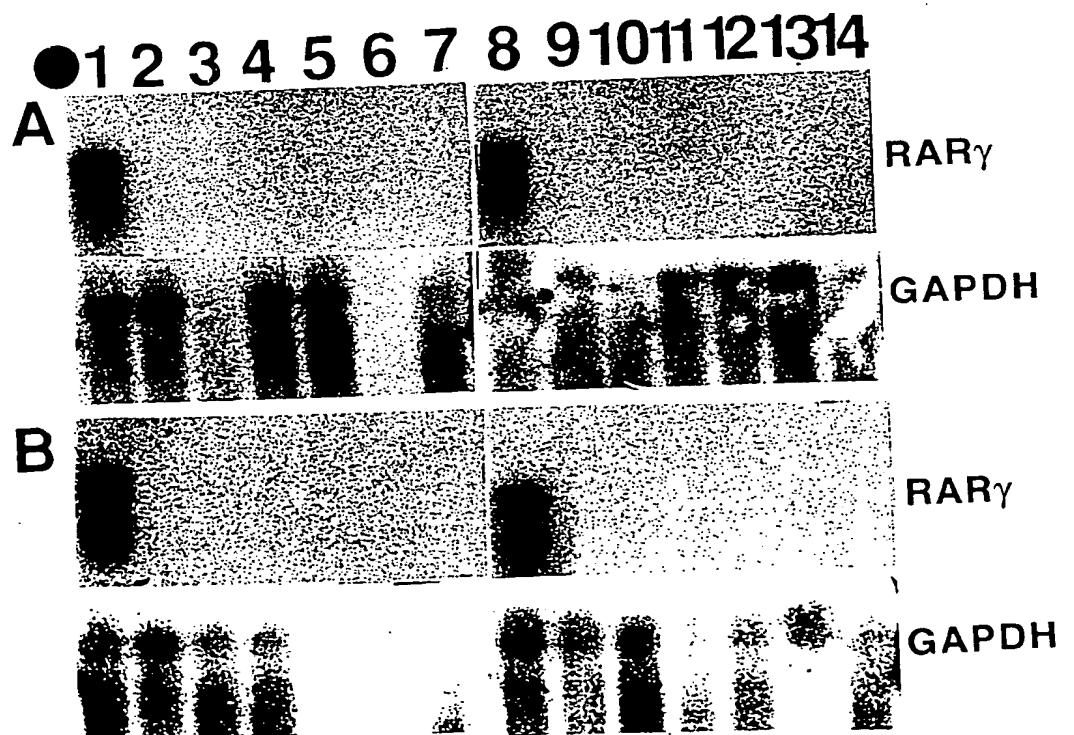
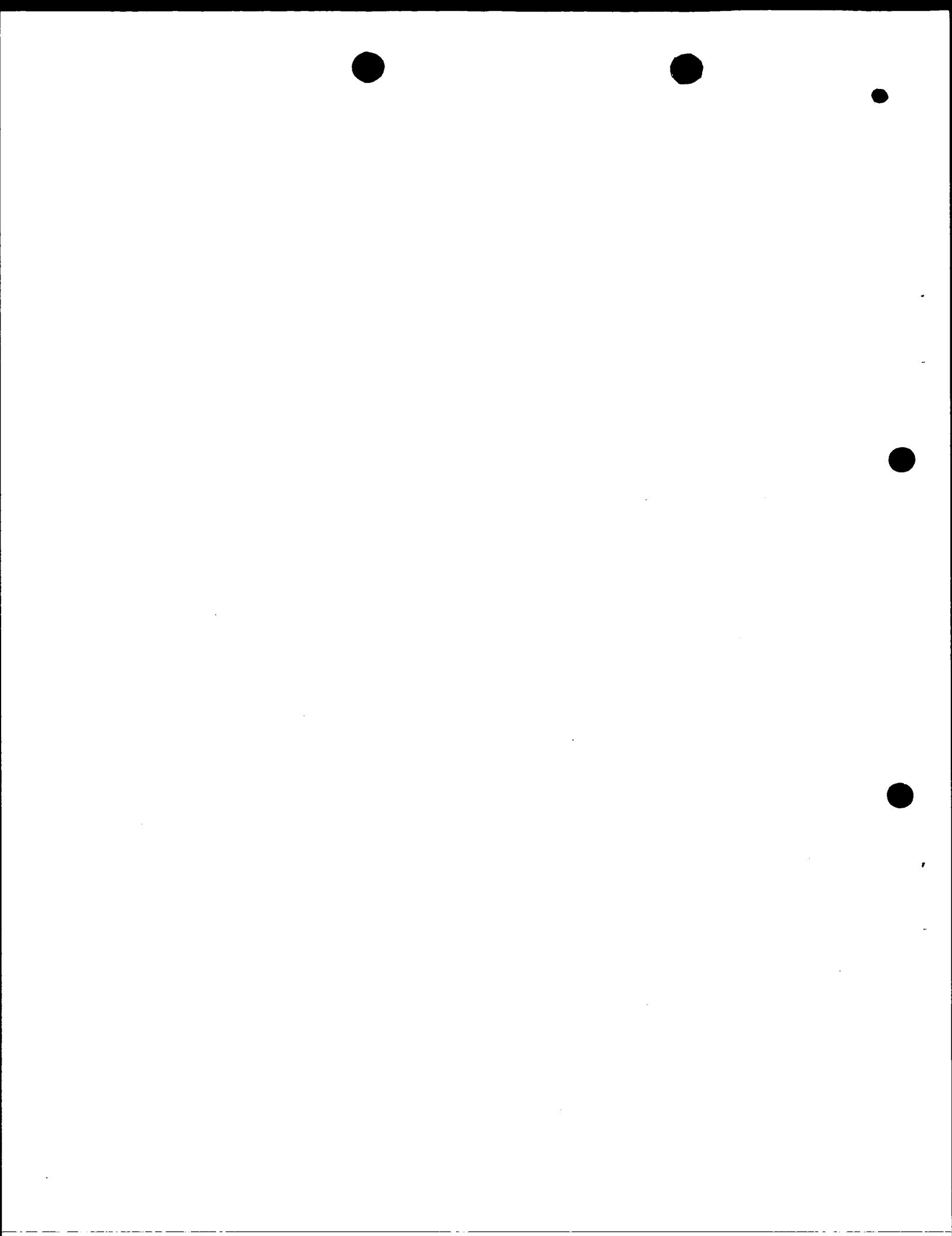


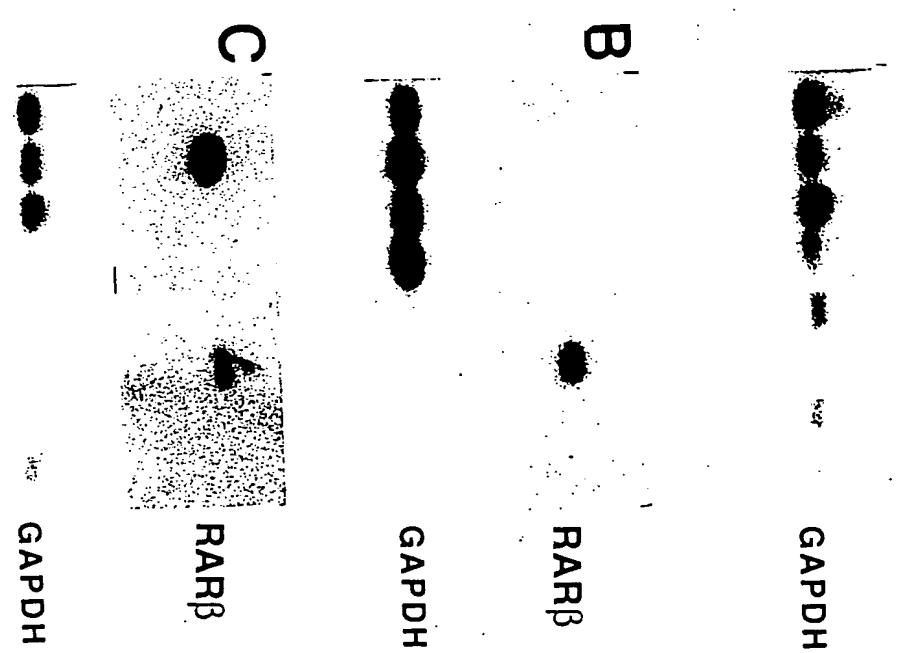
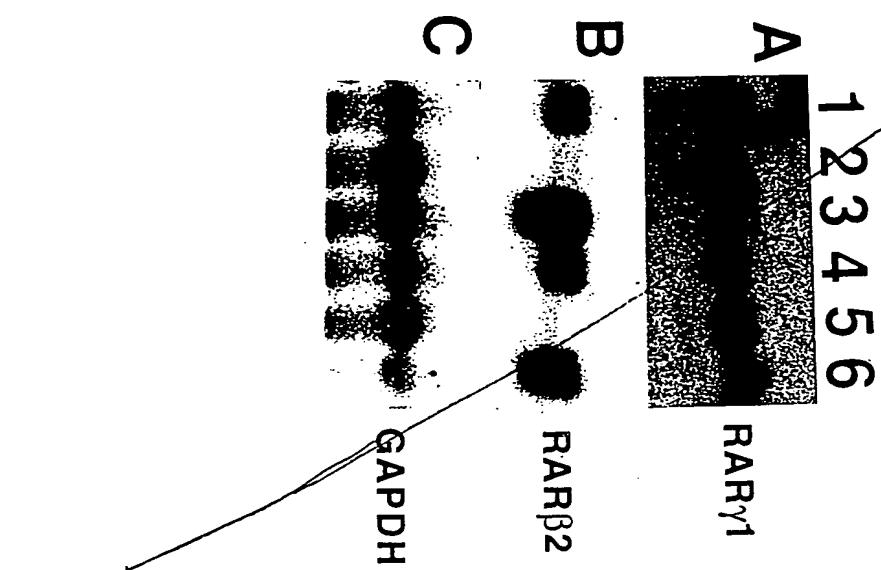
Figure 4





FIGURES.

Fig 7.



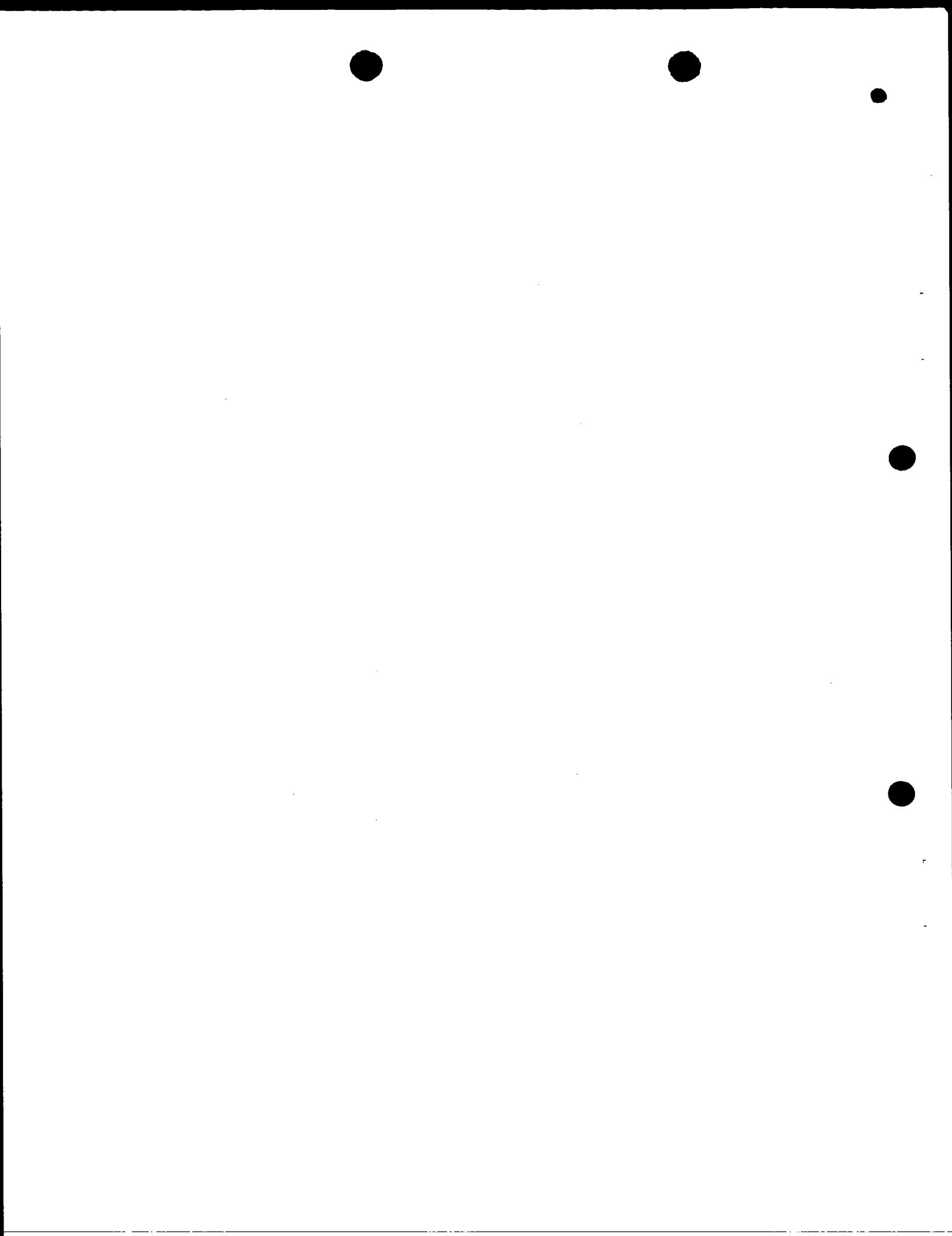
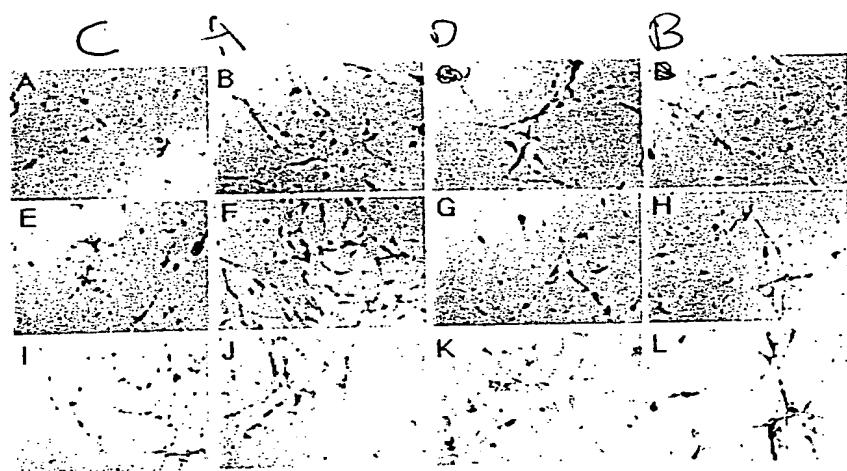
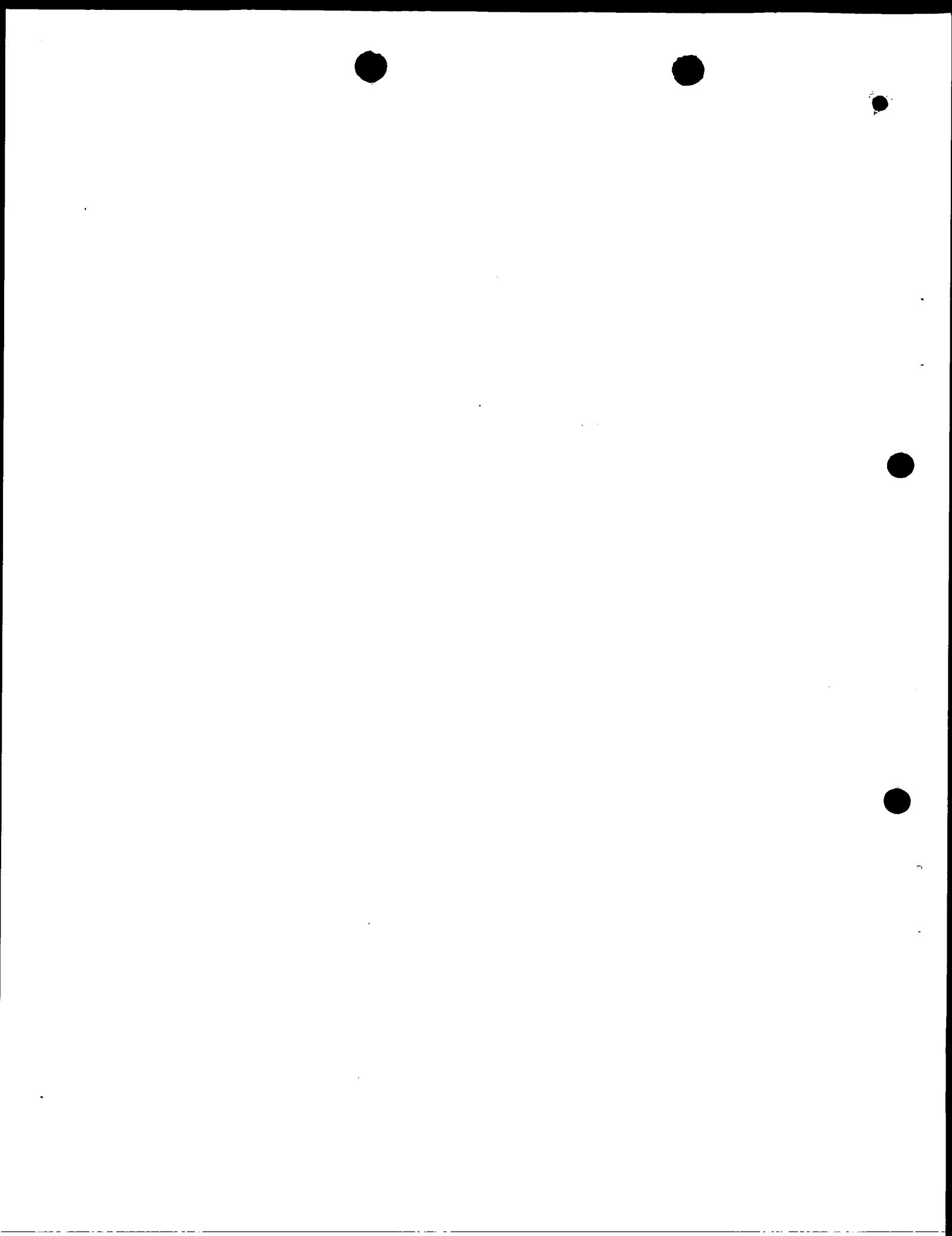


Figure 5.





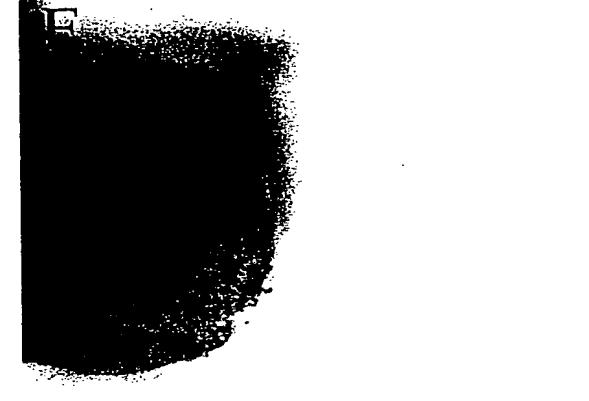
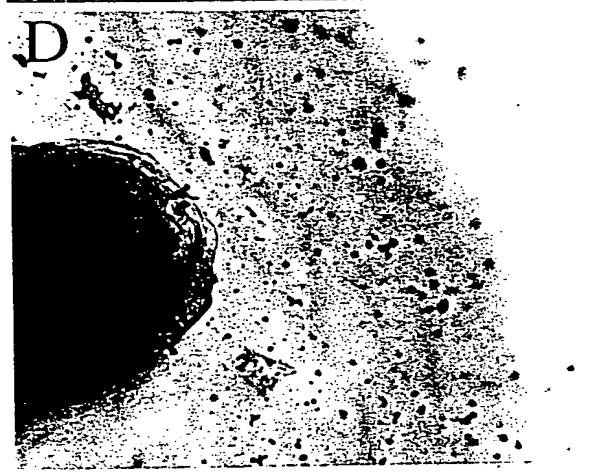
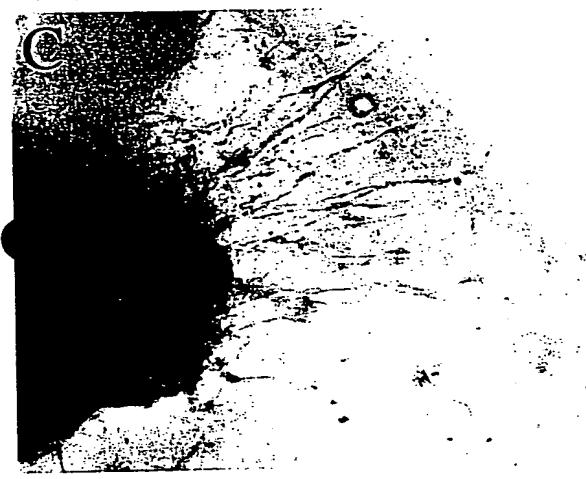
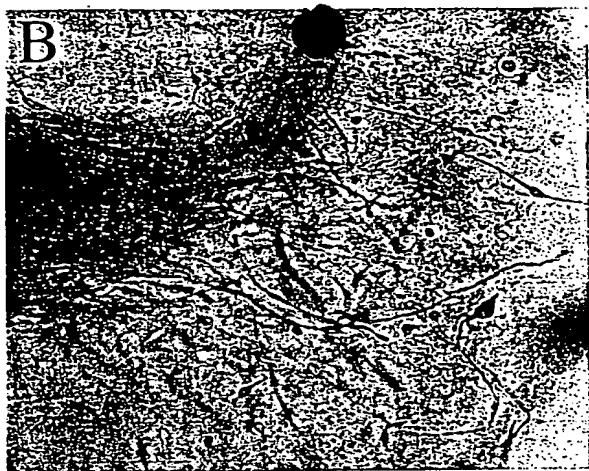
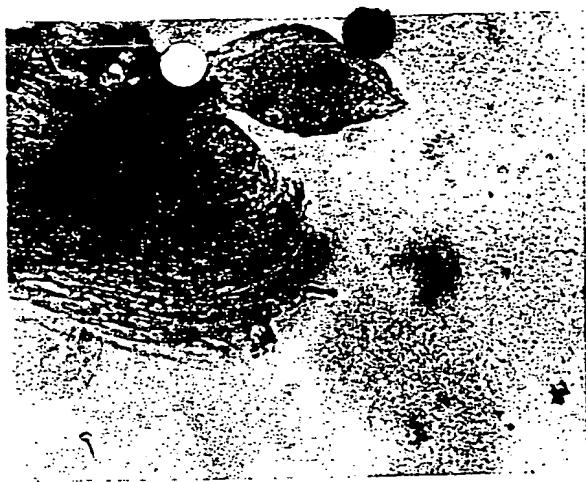
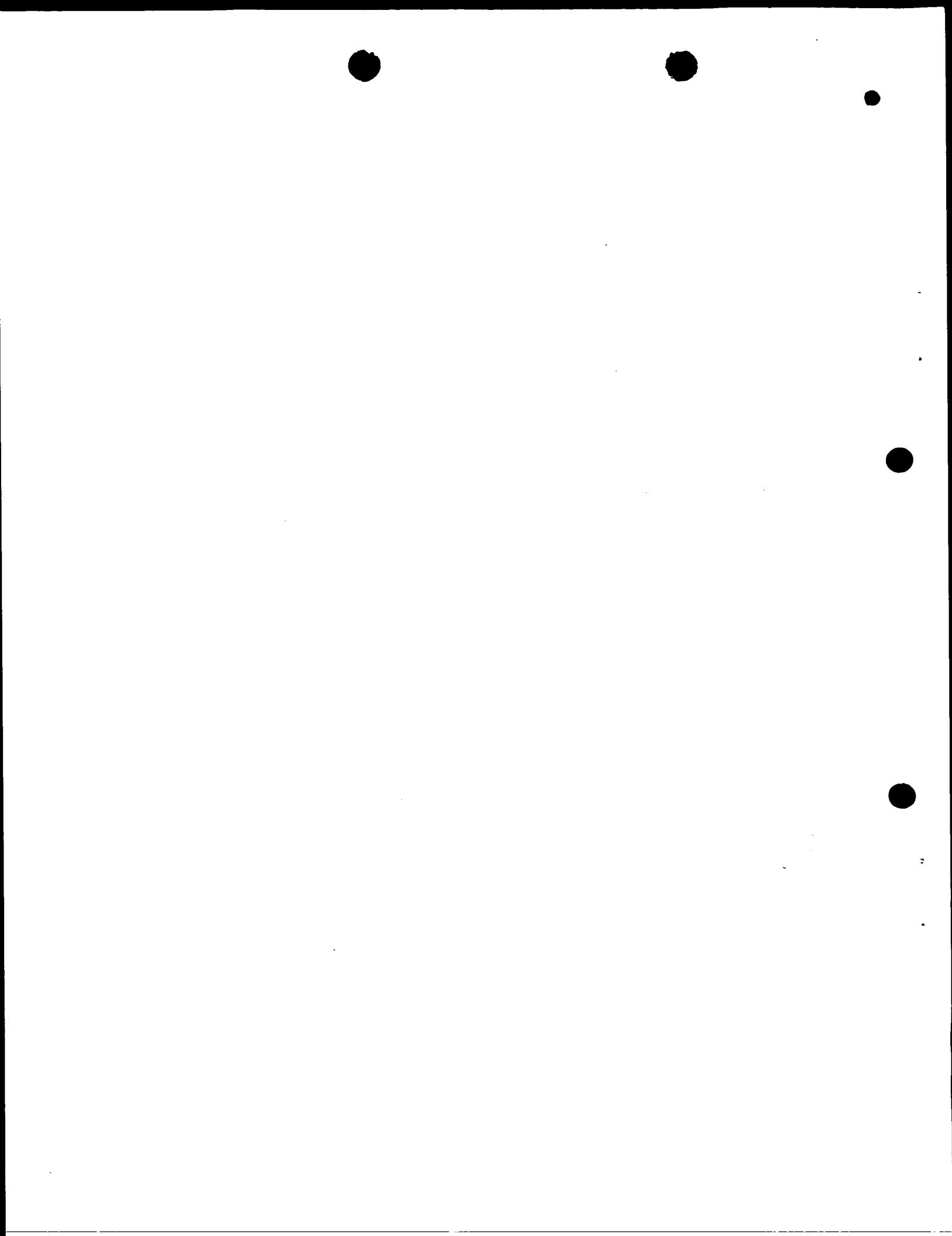
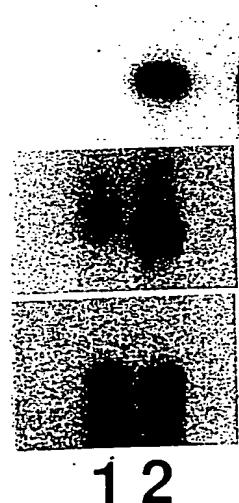
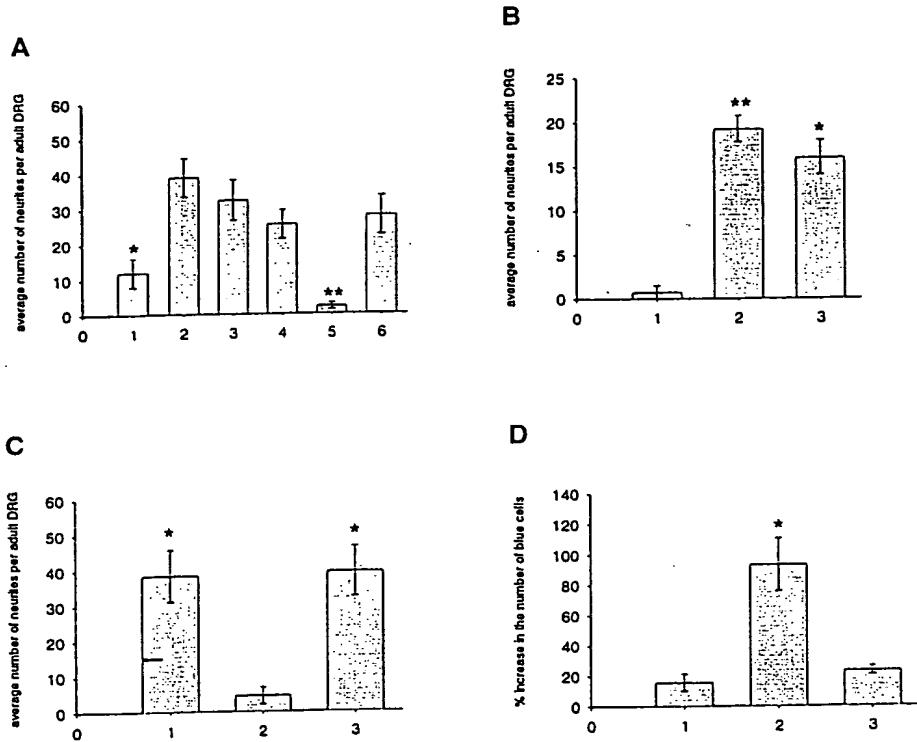


Figure 6.





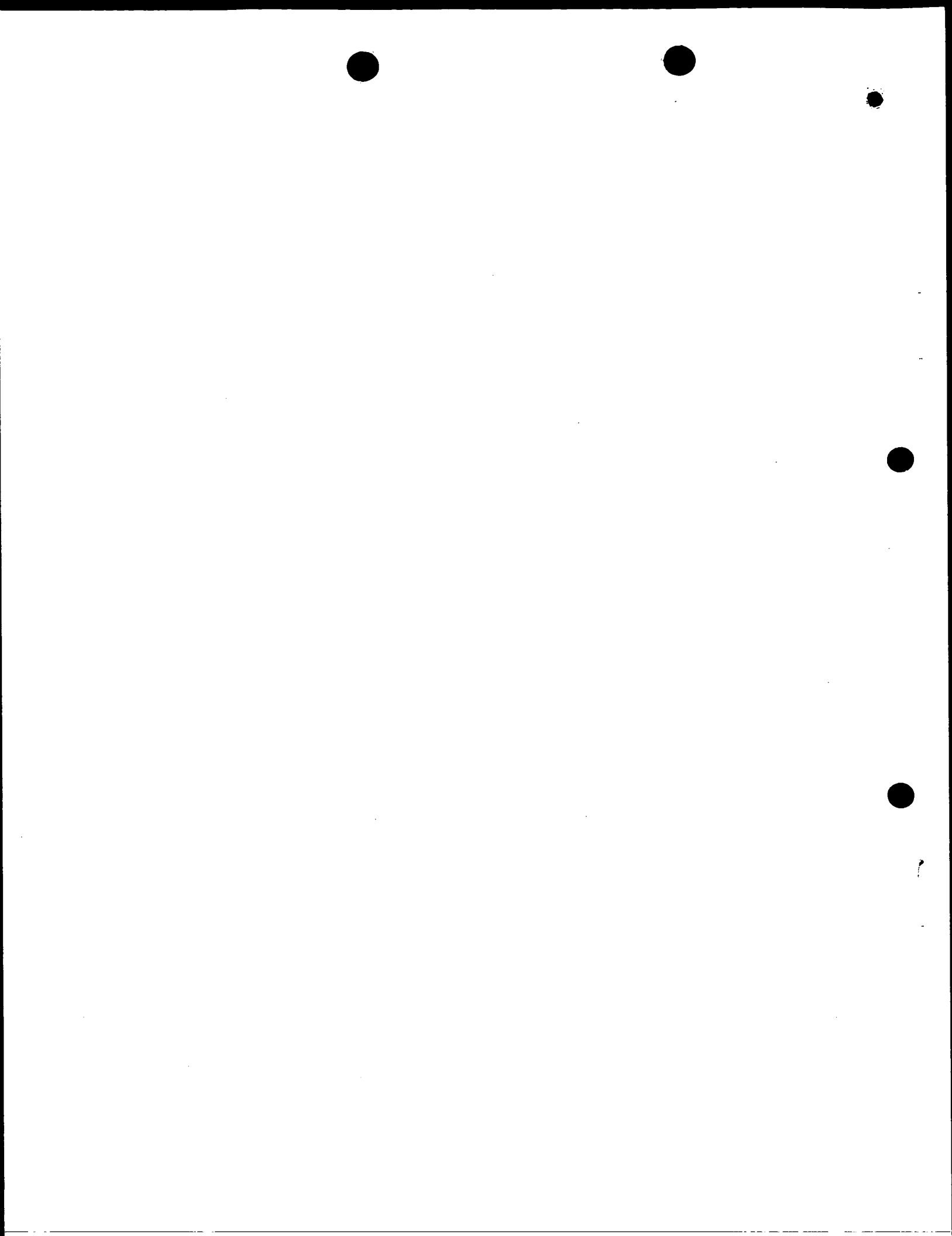
RALDH-2

RAR- $\beta$

GAPDH

1 2

Figure 7.



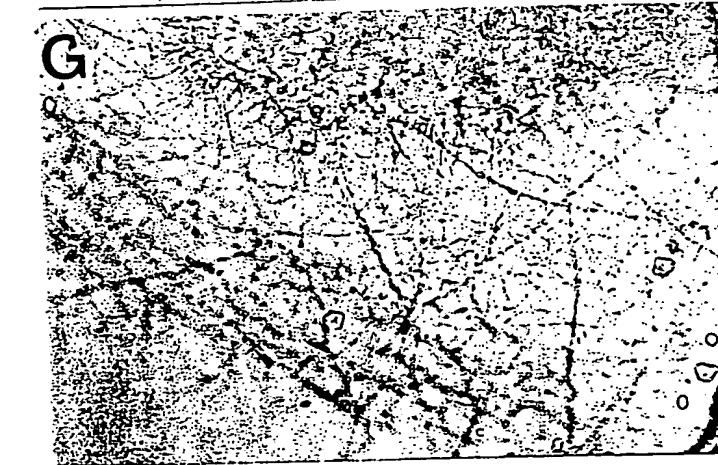
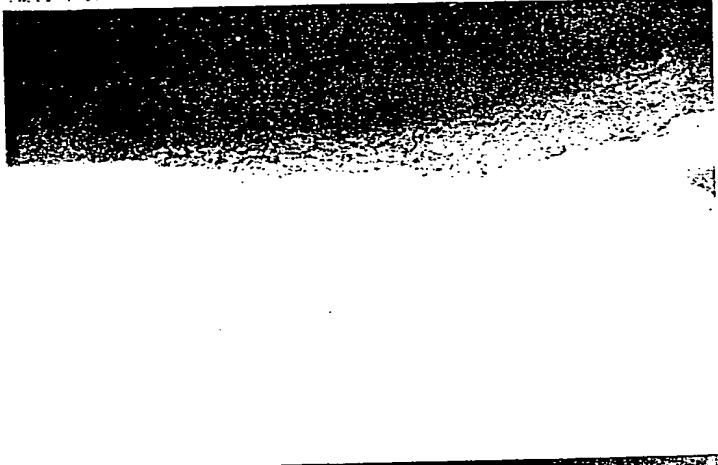
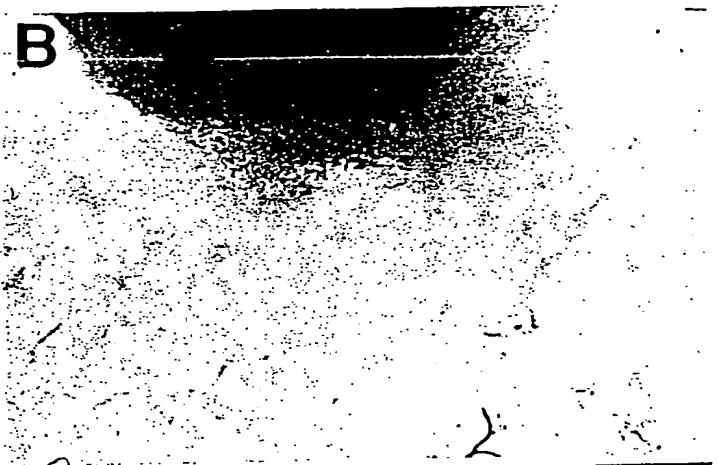
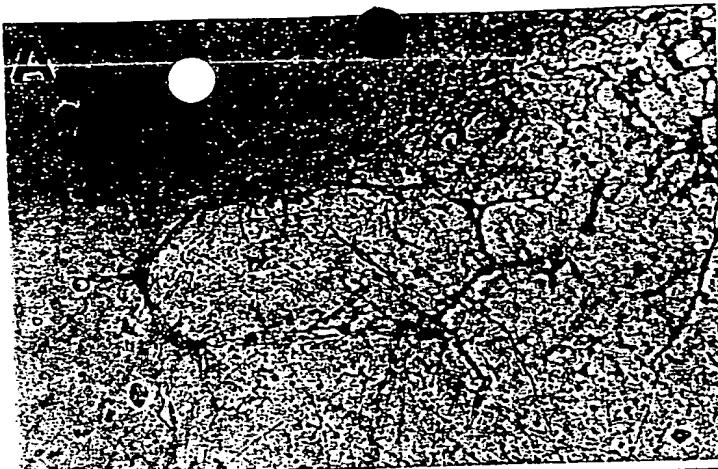


Figure 8.

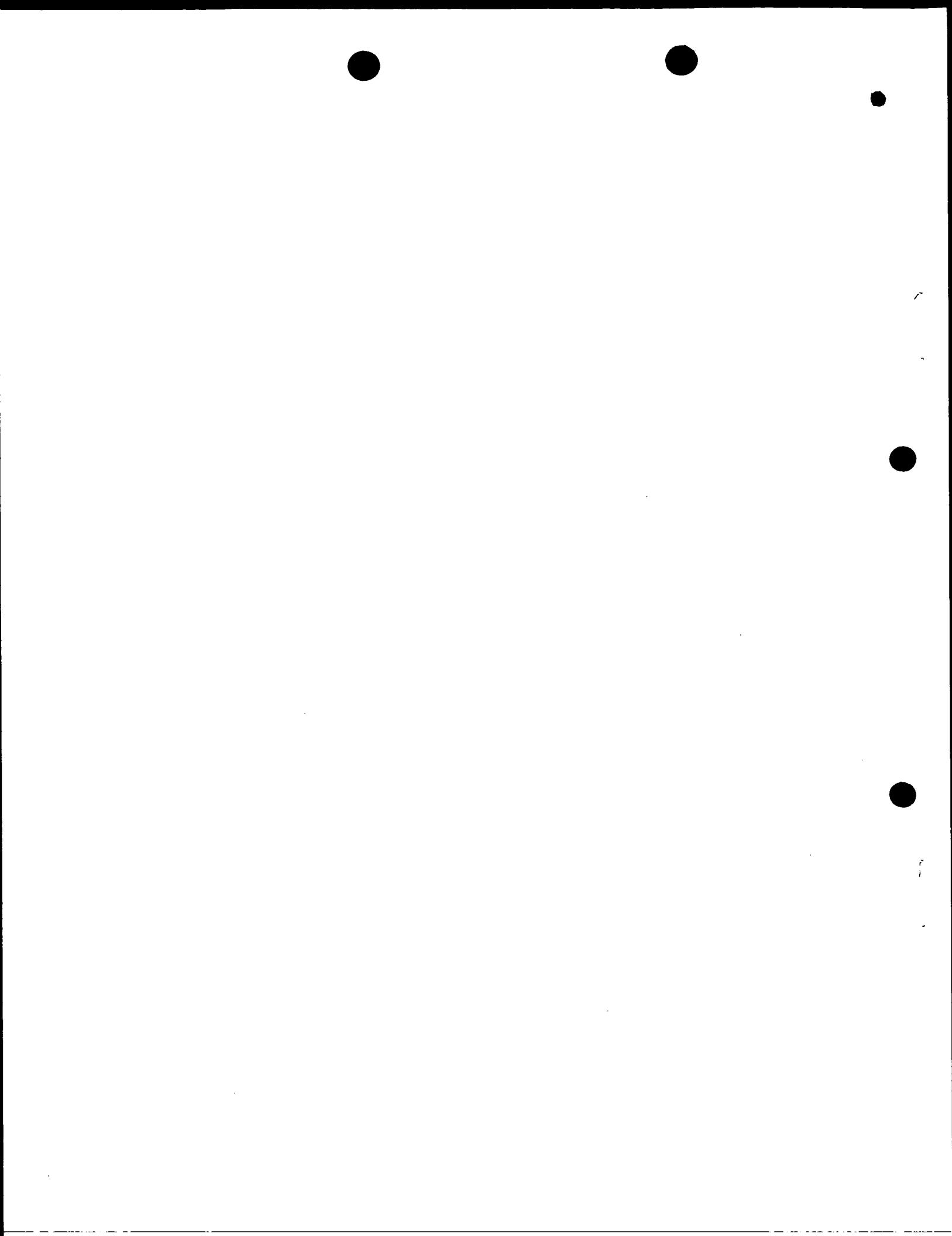


Figure 11.

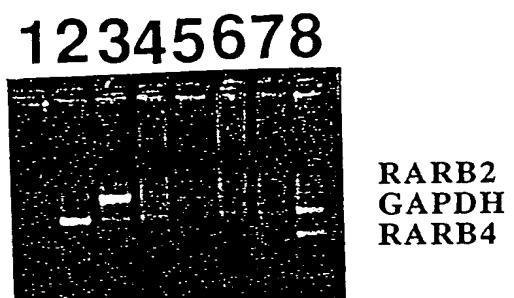
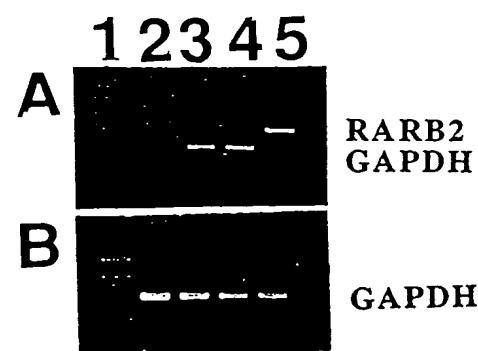
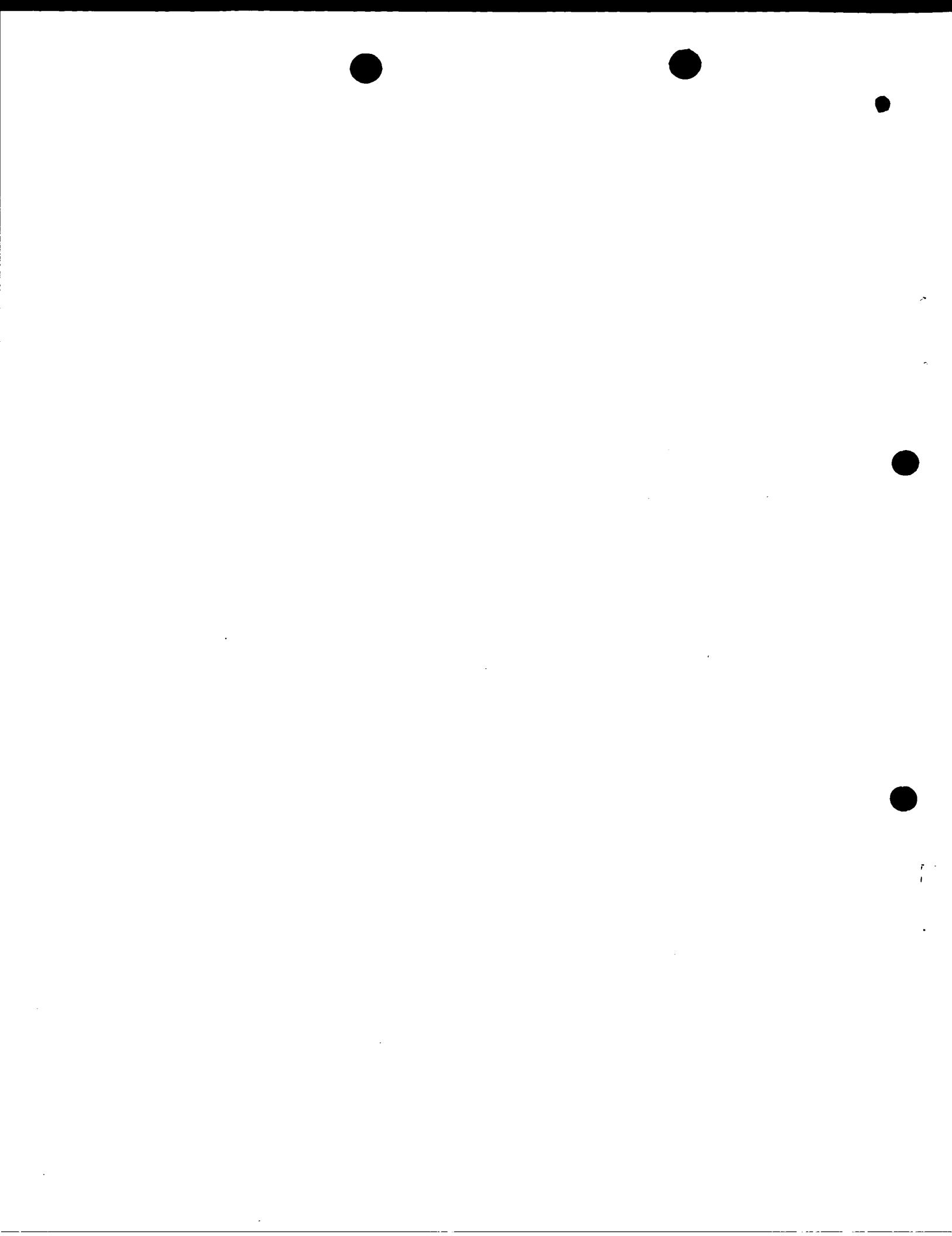


Figure 9.





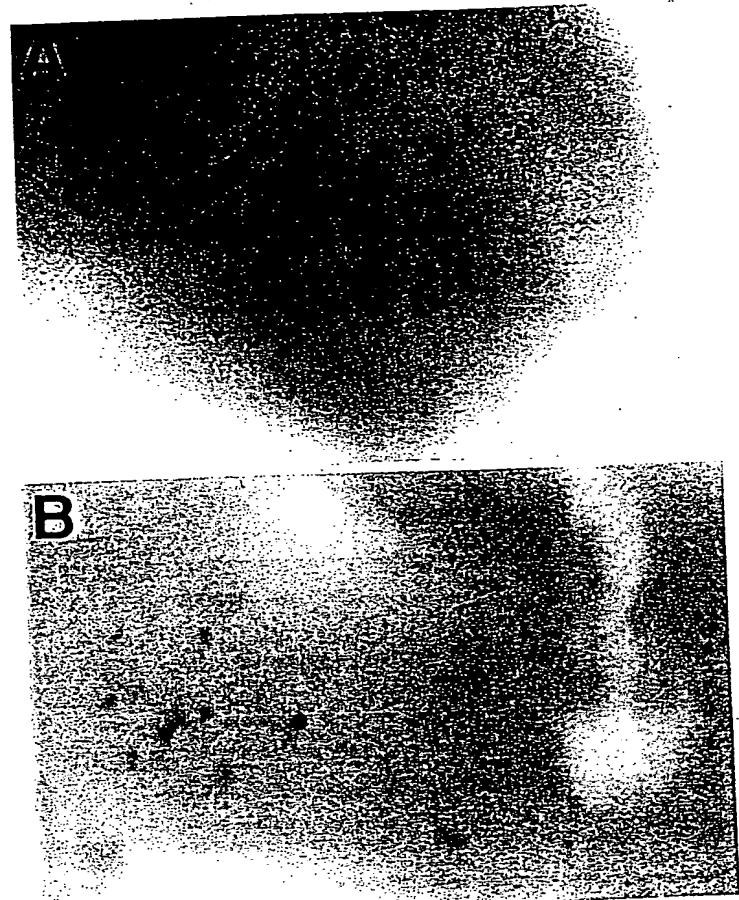
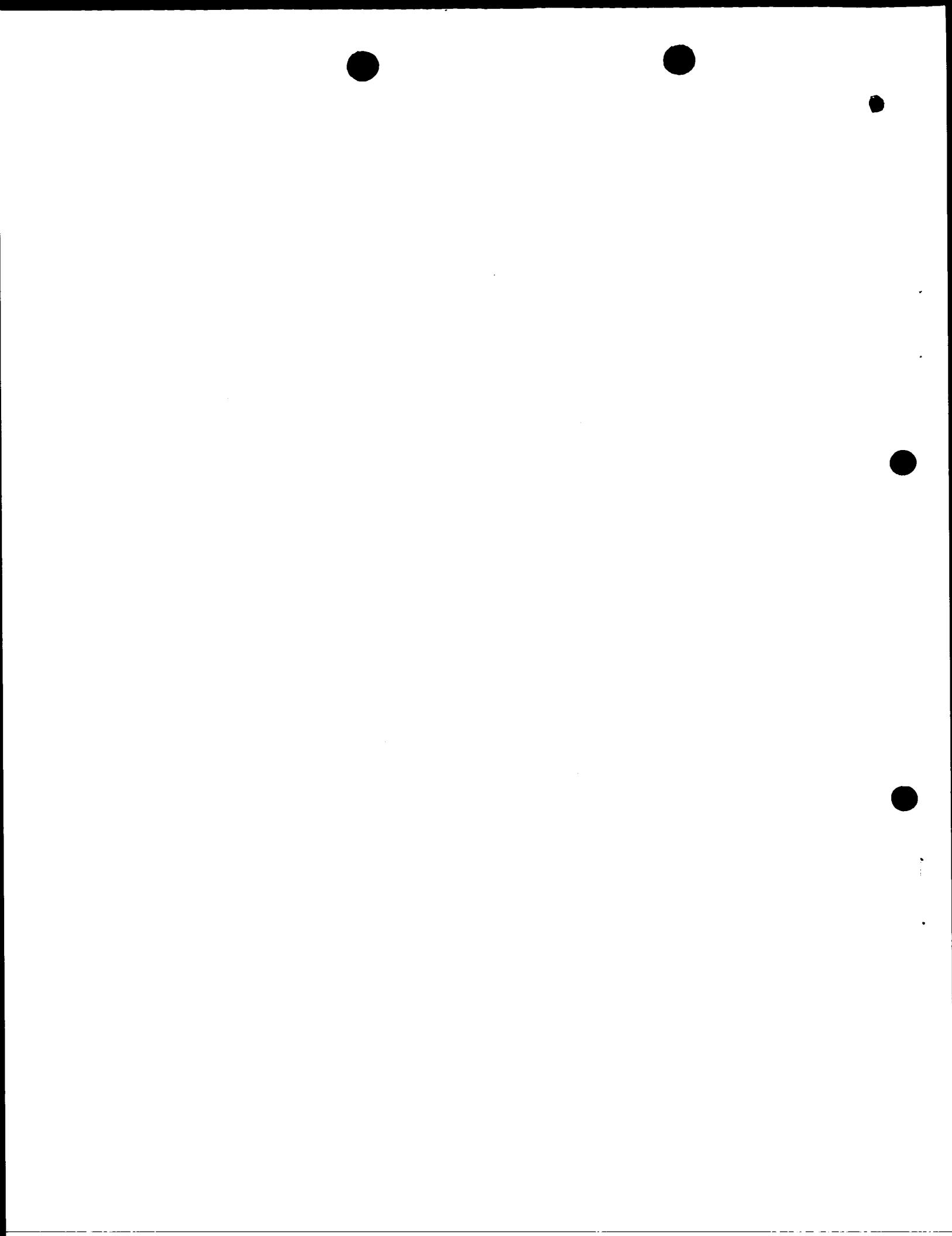


Figure 10.



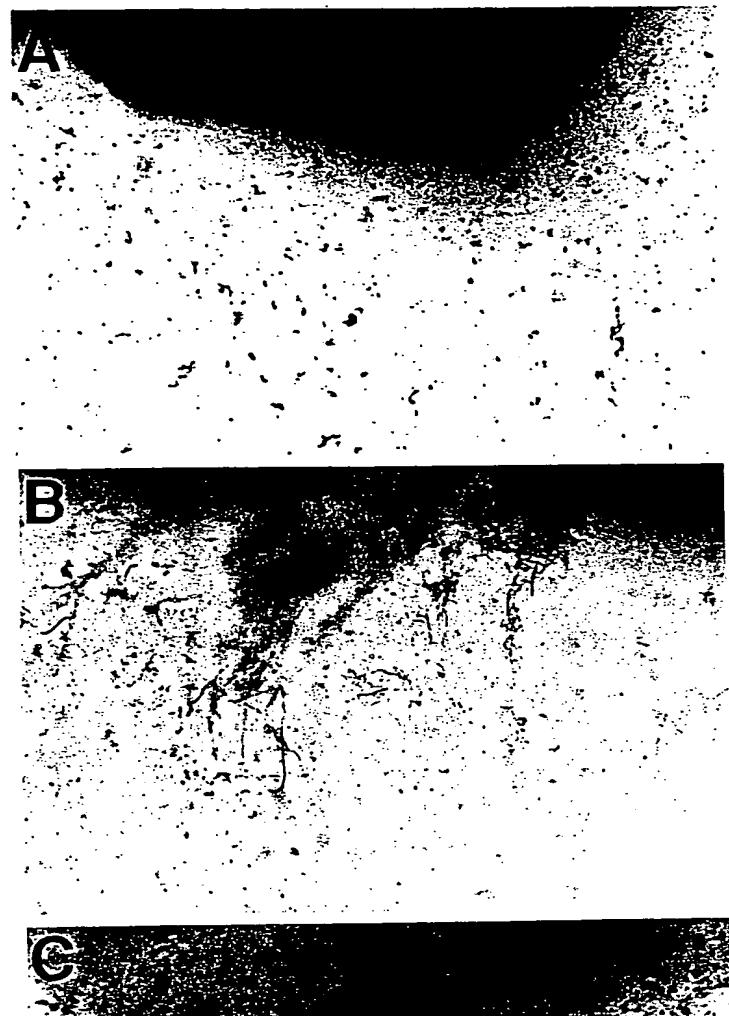


Figure 12

